OIPE JC177
APR 3 0 2004
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I, Janet Hope, BSc (Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English languages, that I have prepared the attached English translation of pages of a German Patent application in the German language with the title:

"Neue für das lysR3-Gen kodierende Nukleotidsequenzen"

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and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

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By: ______________________

Dated: 19th March 2001

New nucleotide sequences which code for the lysR3 gene

The invention provides nucleotide sequences from coryneform bacteria which code for the lysR3 gene and a process for the fermentative preparation of amino acids, in particular L-lysine and L-valine, by attenuation of the lysR3 gene. The lysR3 gene codes for the LysR3 protein, which is a transcription regulator of the LysR family.

Prior art

L-Amino acids, in particular L-lysine and L-valine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acids.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-lysine and L-valine.

Description of the invention

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the lysR3 gene, chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of the transcription regulator LysR3.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

(i) the nucleotide sequence shown in SEQ ID No.1 or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

The invention also provides:

a DNA which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing the polynucleotide according to the invention, point d, in particular pCR2.1lysR3int, deposited in Escherichia coli DSM 13618 at the DSMZ [German Collection of Microorganisms and Cell Cultures], Braunschweig (Germany);

and coryneform bacteria which contain an insertion or deletion in the lysR3 gene, in particular using the vector pCR2.1lysR3int.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library, which comprises the complete gene with the polynucleotide sequence corresponding to SEQ ID No. 1, with a probe which comprises the sequence of the polynucleotide mentioned, according to SEQ ID No. 1 or a fragment thereof, and isolation of the DNA sequence mentioned.

Polynucleotide sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for the LysR3 protein or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the lysR3 gene.

Polynucleotide sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for the LysR3 protein can be prepared with the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of the LysR3 protein, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention moreover provides a process for the fermentative preparation of amino acids, in particular

L-lysine and L-valine, using coryneform bacteria which in particular already produce amino acids, and in which the nucleotide sequences which code for the lysR3 gene are attenuated, in particular eliminated or expressed at a low level.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

The microorganisms which the present invention provides can prepare amino acids, in particular L-lysine and L-valine, from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum (C. glutamicum), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared therefrom, such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DG52-5
Corynebacterium glutamicum DSM 5714 and
Corynebacterium glutamicum DSM 12866

or such as, for example, the L-valine-producing strains
Corynebacterium glutamicum DSM 12455
Corynebacterium glutamicum FERM-P 9325
Brevibacterium lactofermentum FERM-P 9324
Brevibacterium lactofermentum FERM-BP 1763.

The inventors have succeeded in isolating the new lysR3 gene of C. glutamicum which codes for the LysR3 protein, which is a transcription regulator of the LysR family.

To isolate the lysR3 gene or also other genes of C. glutamicum, a gene library of this microorganism is first set up in Escherichia coli (E. coli). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie [Genes and Clones, An Introduction to Genetic Engineering] (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the E. coli K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene

library of C. glutamicum ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the E. coli K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575). Börmann et al. (Molecular Microbiology 6(3), 317-326)) (1992)) in turn describe a gene library of C. glutamicum ATCC13032 using the cosmid pHC79 (Hohn and Collins, 1980, Gene 11, 291-298).

To prepare a gene library of C. glutamicum in E. coli it is also possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable host are, in particular, those E. coli strains which are restriction- and recombination-defective, such as, for example, the strain DH5α (Jeffrey H. Miller: „A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria“, Cold Spring Harbour Laboratory Press, 1992).

The long DNA fragments cloned with the aid of cosmids or other λ-vectors can then be subcloned in turn into the usual vectors suitable for DNA sequencing.

Methods of DNA sequencing are described, inter alia, by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America USA, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of C. glutamicum which codes for the lysR3 gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found in this manner. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the lysR3 gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonukleotide [sic] synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

In the work on the present invention, it has been found that coryneform bacteria produce amino acids, in particular L-lysine and L-valine, in an improved manner after attenuation of the lysR3 gene.

To achieve an attenuation, either the expression of the lysR3 gene or the catalytic properties of the enzyme protein can be reduced or eliminated. The two measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in the patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik

[Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms [Threonine dehydratase from Corynebacterium glutamicum: Cancelling the allosteric regulation and structure of the enzyme]", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summarizing descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions, insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, missense mutations or nonsense mutations are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and Clones]", VCH

Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik [General Genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

A common method of mutating genes of C. glutamicum is the method of gene disruption and gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically E. coli), but not in C. glutamicum. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the central part of the coding region of the gene is then transferred into the desired strain of C. glutamicum by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event, the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick

et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to eliminate the recA gene of C. glutamicum.

Figure 1 shows by way of example the plasmid vector pCR2.1lysR3int, with the aid of which the lysR3 gene can be disrupted or eliminated.

In the method of gene replacement, a mutation, such as e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for C. glutamicum and this is then transferred into the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al.(Microbiology 144, 915 - 927 (1998)) to eliminate the pyc gene of C. glutamicum by a deletion.

A deletion, insertion or a base exchange can be incorporated into the lysR3 gene in this manner.

In addition, it may be advantageous for the production of L-amino acids, in particular L-lysine and L-valine, to enhance, in particular to over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate cycle or of amino acid export, in addition to attenuation of the lysR3 gene.

Thus, for example, for the preparation of L-lysine, at the same time one or more of the genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),

- the eno gene which codes for enolase (DE: 19947791.4),
- the zwf gene which codes for the zwf gene product (JP-A-09224661),
- the pyc gene which codes for pyruvate carboxylase (Peters-Wendisch et al.(Microbiology 144, 915 - 927 (1998))
- the lysE gene which codes for lysine export (DE-A-195 48 222)

can be enhanced, in particular over-expressed.

It may furthermore be advantageous for the production of amino acids, in particular L-lysine, in addition to the attenuation of the lysR3 gene, at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase(US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114)

to be attenuated.

Thus, for example, for the production of L-valine

- at the same time the ilvBN gene which codes for acetohydroxy-acid synthase (Keilhauer et al., (1993) Journal of Bacteriology 175: 5595-5603), or
- at the same time the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979), or

- at the same time the mqo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998))

can be over-expressed.

In addition to attenuation of the lysR3 gene it may furthermore be advantageous, for the production of amino acids, in particular L-lysine and L-valine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids, in particular L-lysine and L-valine. A summary of known culture methods are [sic] described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut

fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for

example, air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli strain TOP10F/pCR2.1lysR3int as DSM 13618.

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine and L-valine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from Escherichia coli and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA). Methods for transformation of Escherichia coli are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

Example 1

Preparation of a genomic cosmid gene library from C. glutamicum ATCC 13032

Chromosomal DNA from C. glutamicum ATCC 13032 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al., 1987, Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Res. 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2

Isolation and sequencing of the lysR3 gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture

being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 μg/ml zeocin.

The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems(Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZerol derivatives were assembled to a continuous contig. The computer-assisted coding region analysis [sic] were prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231). Further analyses were carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research, 25:3389-3402) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading

frame of 633 base pairs, which was called the lysR3 gene. The lysR3 gene codes for a polypeptide of 210 amino acids.

Example 3

Preparation of an integration vector for integration mutagenesis of the lysR3 gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the lysR3 gene known for C. glutamicum from example 2, the following oligonucleotides were chosen for the polymerase chain reaction:

lysR3intA:
5`GAT GTG GTG TTG ATG GAT CT 3`
lysR3intB:
5`TCA ATT TCT CTG GCA CTG AG 3`

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the polymerase chain reaction, an internal fragment of the lysR3 gene 323 bp in size was isolated, this being shown in SEQ ID No. 3.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead at al. (1991) Bio/Technology 9:657-663).

The E. coli strain TOP10F was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol. I, IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook

et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1lysR3int.

<u>Example 4</u>

Integration mutagenesis of the lysR3 gene in the lysine producer DSM 5715 and in the valine producer FERM BP-1763

The vector pCR2.1lysR3int mentioned in example 3 was electroporated by the electroporation method of Tauch et al.(FEMS Microbiological Letters, 123:343-347 (1994)) into Corynebacterium glutamicum DSM 5715 and Brevibacterium lactofermentum FERM BP-1763. The strain DSM 5715 is an AEC-resistant lysine producer. The strain FERM BP-1763 is a valine producer in need of isoleucine and methionine. The vector pCR2.1lysR3int cannot replicate independently in DSM 5715 or FERM BP-1763 and is retained in the cell only if it has integrated into the chromosome of DSM 5715 or FERM BP-1763. Selection of clones with pCR2.1lysR3int integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin.

For detection of the integration, the lysR3int fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the

restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybrization [sic] kit from Boehringer. The plasmid pCR2.1lysR3int mentioned in example 3 had been inserted into the chromosome of DSM5715 and FERM BP-1763 within the chromosomal lysR3 gene. The strains were called DSM5715::pCR2.1lysR3int and FERM BP-1763::pCR2.1lysR3int.

Example 5

Preparation of L-lysine and L-valine

The C. glutamicum and B. lactofermentum strains DSM5715::pCR2.1lysR3int and FERM BP-1763::pCR2.1lysR3int obtained in example 4 were cultured in a nutrient medium suitable for the production of L-lysine and L-valine and the L-lysine and L-valine content in the culture supernatant was determined.

For this, the strains were first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Kanamycin (25 mg/l) was added to this. The preculture was incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1 OD. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
$(NH_4)_2SO_4)$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions are then added, as well as the $CaCO_3$ autoclaved in the dry state. For culturing of DSM 5715, 0.1 g/l leucine was additionally added to the medium. For culturing of FERM BP-1763, 0.1 g/l isoleucine and 0.1 g/l methionine were additionally added to the medium.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of L-lysine and of L-valine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The results of the experiment are shown in tables 1 and 2.

Table 1

Strain	OD(660)	Lysine HCl g/l
DSM5715	7.5	13.01
DSM5715::pCR2.1lysR3int	7.6	15.04

Table 2

Strain	OD(660)	Valine g/l
FERM BP-1763	12.1	7.49
FERM BP-1763::pCR2.1lysR3int	12.5	8.67

SEQUENCE PROTOCOL

<110> Degussa-Hüls AG

<120> New nucleotide sequences which code for the lysR3 gene

<130> 000183 BT

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<160> 3

<170> PatentIn Ver. 2.1

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<223> lysR3 gene

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acaataatta ttgaatctac aatcggatcg ggaactggaa tttccgcccg ttttccctat 180

ccacaaaagg accaagataa gtg atc cgt att ctg ttg gct gat gat cat ccc 233
                      Met Ile Arg Ile Leu Leu Ala Asp Asp His Pro
                        1               5                   10

gtt gtt cgc gca ggc ctt gcc tcc ttg ctg gtg agt gaa gat gat ttt   281
Val Val Arg Ala Gly Leu Ala Ser Leu Leu Val Ser Glu Asp Asp Phe
            15                  20                  25

gag ata gtg gac atg gtg ggc acc cca gat gat gcc gtt gcg cgc gcc   329
Glu Ile Val Asp Met Val Gly Thr Pro Asp Asp Ala Val Ala Arg Ala
        30                  35                  40

gcg gaa ggc ggg gtg gat gtg gtg ttg atg gat ctg cgt ttt ggt gat   377
Ala Glu Gly Gly Val Asp Val Val Leu Met Asp Leu Arg Phe Gly Asp
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caa cca ggc atc gag gtc gcc ggc ggg gta gag gca acg cgt cgc atc   425
Gln Pro Gly Ile Glu Val Ala Gly Gly Val Glu Ala Thr Arg Arg Ile
 60                  65                  70                  75

cgt gcg ctg gac aac ccg cca cag gta ctg gtg gtg acc aac tac tcc   473
Arg Ala Leu Asp Asn Pro Pro Gln Val Leu Val Val Thr Asn Tyr Ser
                 80                  85                  90

aca gac ggc gat gtg gtg ggc gca gta tct gct ggt gcc gtg ggg tat   521
Thr Asp Gly Asp Val Val Gly Ala Val Ser Ala Gly Ala Val Gly Tyr
             95                 100                 105
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        110                 115                 120

gcc gcg cgg gga gaa tca gtg ctt tca aag cag gtc gcc agc aag atc    617
Ala Ala Arg Gly Glu Ser Val Leu Ser Lys Gln Val Ala Ser Lys Ile
    125                 130                 135

atg ggg cgg atg aac aac ccc atg act gct ctc agt gcc aga gaa att    665
Met Gly Arg Met Asn Asn Pro Met Thr Ala Leu Ser Ala Arg Glu Ile
140                 145                 150                 155

gaa gtg ctg tcc ttg gtg gcg caa ggg caa agc aat aga gaa atc ggc    713
Glu Val Leu Ser Leu Val Ala Gln Gly Gln Ser Asn Arg Glu Ile Gly
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aag aaa ctt ttc ctc act gag gcc acg gtg aaa agt cac atg ggg cat    761
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gtg ttc aac aag ctg gat gtc acc tct aga aca gct gcg gta gct gaa    809
Val Phe Asn Lys Leu Asp Val Thr Ser Arg Thr Ala Ala Val Ala Glu
        190                 195                 200

gcc aga cag cgc gga att atc tagacgcaca cgtgttggta accgatcaca      860
Ala Arg Gln Arg Gly Ile Ile
    205                 210

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ttgtgcatga cggtggcatt tgctggagga agcctgaccg cgtgcacacc tcgtcctgat 980

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&lt;210&gt; 2
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&lt;400&gt; 2
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 1               5                  10                  15

Leu Ala Ser Leu Leu Val Ser Glu Asp Asp Phe Glu Ile Val Asp Met
            20                  25                  30

Val Gly Thr Pro Asp Asp Ala Val Ala Arg Ala Ala Glu Gly Gly Val
        35                  40                  45

Asp Val Val Leu Met Asp Leu Arg Phe Gly Asp Gln Pro Gly Ile Glu
    50                  55                  60

Val Ala Gly Gly Val Glu Ala Thr Arg Arg Ile Arg Ala Leu Asp Asn
65                  70                  75                  80

Pro Pro Gln Val Leu Val Val Thr Asn Tyr Ser Thr Asp Gly Asp Val
                85                  90                  95
```

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Val Gly Ala Val Ser Ala Gly Ala Val Gly Tyr Leu Leu Lys Asp Ser
            100                 105                 110

Ser Pro Glu Asp Leu Ile Ala Gly Val Arg Asp Ala Ala Arg Gly Glu
        115                 120                 125

Ser Val Leu Ser Lys Gln Val Ala Ser Lys Ile Met Gly Arg Met Asn
    130                 135                 140

Asn Pro Met Thr Ala Leu Ser Ala Arg Glu Ile Glu Val Leu Ser Leu
145                 150                 155                 160

Val Ala Gln Gly Gln Ser Asn Arg Glu Ile Gly Lys Lys Leu Phe Leu
                165                 170                 175

Thr Glu Ala Thr Val Lys Ser His Met Gly His Val Phe Asn Lys Leu
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Ile Ile
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ctcaaagata gctccccaga agatctcatt gccggtgttc gcgatgccgc gcggggagaa 240
tcagtgcttt caaagcaggt cgccagcaag atcatggggc ggatgaacaa ccccatgact 300
gctctcagtg ccagagaaat tga                                         323
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The following figures are attached:
Figure 1: Map of the plasmid pCR2.1lysR3int.

The abbreviations and designations used have the following meaning.

KmR:	Kanamycin resistance gene
EcoRI:	cleavage site of the restriction enzyme EcoRI
lysR3int:	Internal fragment of the lysR3 gene
ColE1 ori:	Replication origin of the plasmid ColE1

Patent claims

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the lysR3 gene, chosen from the group consisting of

   a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

   b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least to [sic] 70% to the amino acid sequence of SEQ ID No. 2,

   c) polynucleotide which is complementary to the polynucleotides of a) or b), and

   d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b), or c),

   the polypeptide preferably having the activity of the transcription regulator LysR3.

2. A polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.

3. A polynucleotide as claimed in claim 1, wherein the polynucleotide is an RNA.

4. A DNA as claimed in claim 2 which is capable of replication, comprising

   (i) the nucleotide sequence shown in SEQ ID no. 1, or

(ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or

(iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

5. A polynucleotide as claimed in claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.

6. A coryneform bacterium in which the lysR3 gene is attenuated, preferably eliminated.

7. A process for the preparation of L-amino acids, in particular L-lysine and L-valine,
which comprises
carrying out the following steps,
a) fermentation of the bacteria which produce the desired L-amino acid and in which at least the lysR3 gene is attenuated,
b) concentration of the desired product in the medium or in the cells of the bacteria and
c) isolation of the L-amino acid.

8. A process as claimed in claim 7,
wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

9. A process as claimed in claim 7,
wherein
bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.

10. A process as claimed in claim 7, wherein expression of the polynucleotide(s) which codes (code) for the lysR3 gene is decreased, in particular eliminated.

11. A process as claimed in claim 7, wherein the regulatory properties of the polypeptide for which the polynucleotide lysR3 codes are decreased.

12. A process as claimed in claim 7, wherein for the preparation of L-amino acids, in particular L-lysine, bacteria in which at the same time one of the genes chosen from the group consisting of

12.1 the dapA gene which codes for dihydrodipicolinate synthase,

12.2 the eno gene which codes for enolase,

12.3 the zwf gene which codes for the zwf gene product,

12.4 the pyc gene which codes for pyruvate carboxylase,

12.5 the lysE gene which codes for lysine export,

is or are enhanced, preferably over-expressed, are fermented.

13. A process as claim in claim 7, wherein at the same time one or more of the genes chosen from the group consisting of:

13.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,

13.2 the pgi gene which codes for glucose 6-phosphate isomerase,

13.3 the poxB gene which codes for pyruvate oxidase is or are attenuated.

14. A process as claimed in claim 7, wherein for the preparation of L-amino acids, in particular L-valine, bacteria in which at the same time one or more of the genes chosen from the group consisting of

14.1 the ilvBN gene which codes for acetohydroxy-acid synthase,

14.2 the ilvD gene which codes for dihydroxy-acid dehydratase,

14.3 the mqo gene which codes for malate:quinone oxidoreductase is or are attenuated are fermented.

15. A process as claimed in one or more of the preceding claims, wherein microorganisms of the genus Corynebacterium glutamicum or Brevibacterium lactofermentum are employed.

16. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for the transcription regulator LysR3 or have a high similarity to the sequence of the lysR3 gene, which comprises employing the polynucleotide sequences as claimed in claims 1 to 4 as hybridization probes.

New nucleotide sequences which code for the lysR3 gene

Abstract

Isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of

a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,

b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

c) polynucleotide which is complementary to the polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the fermentative preparation of L-amino acids using coryneform bacteria, in which at least the lysR3 gene is present in attenuated form, and the use of the polynucleotide sequences as hydribization probes.

Figure 1: Plasmid map of pCR2.1lysR3int